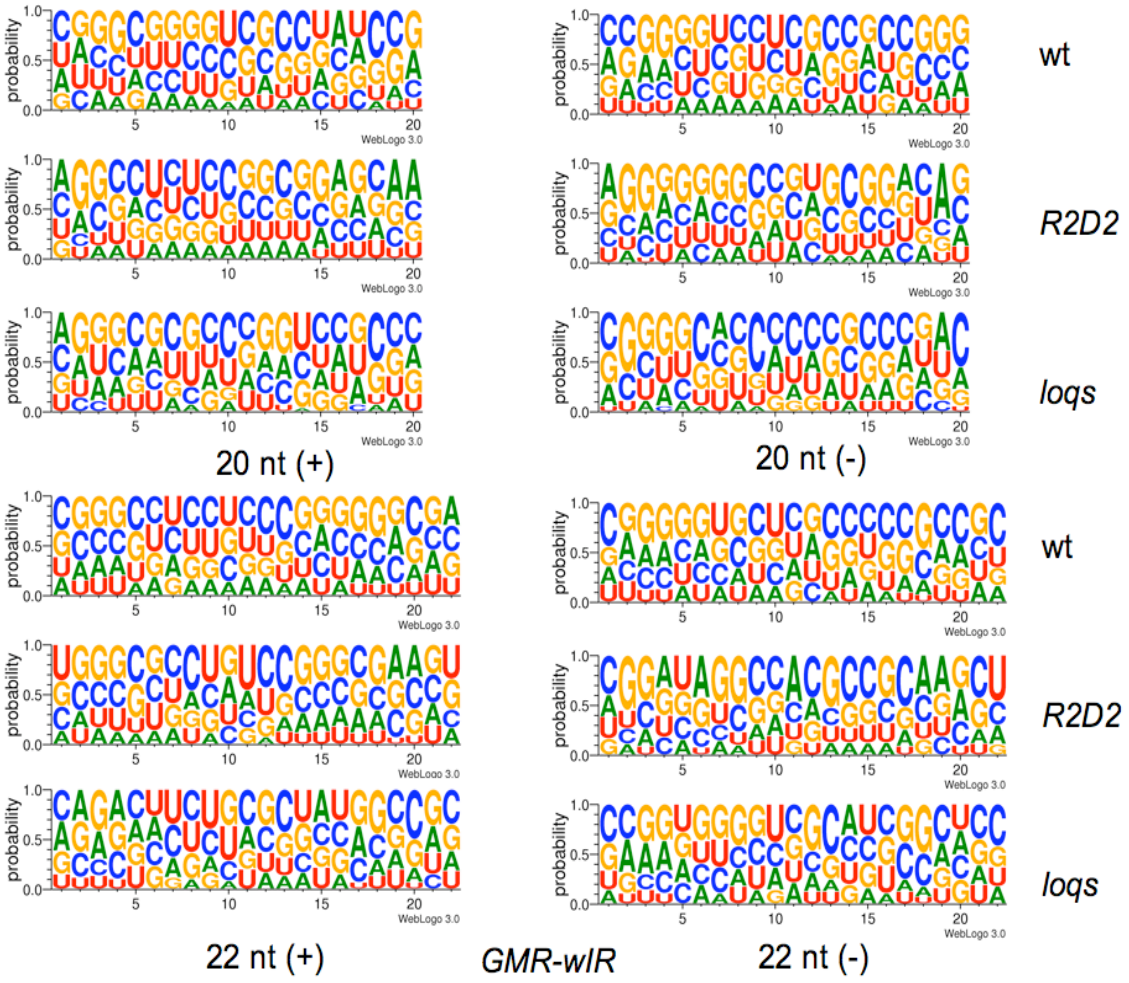
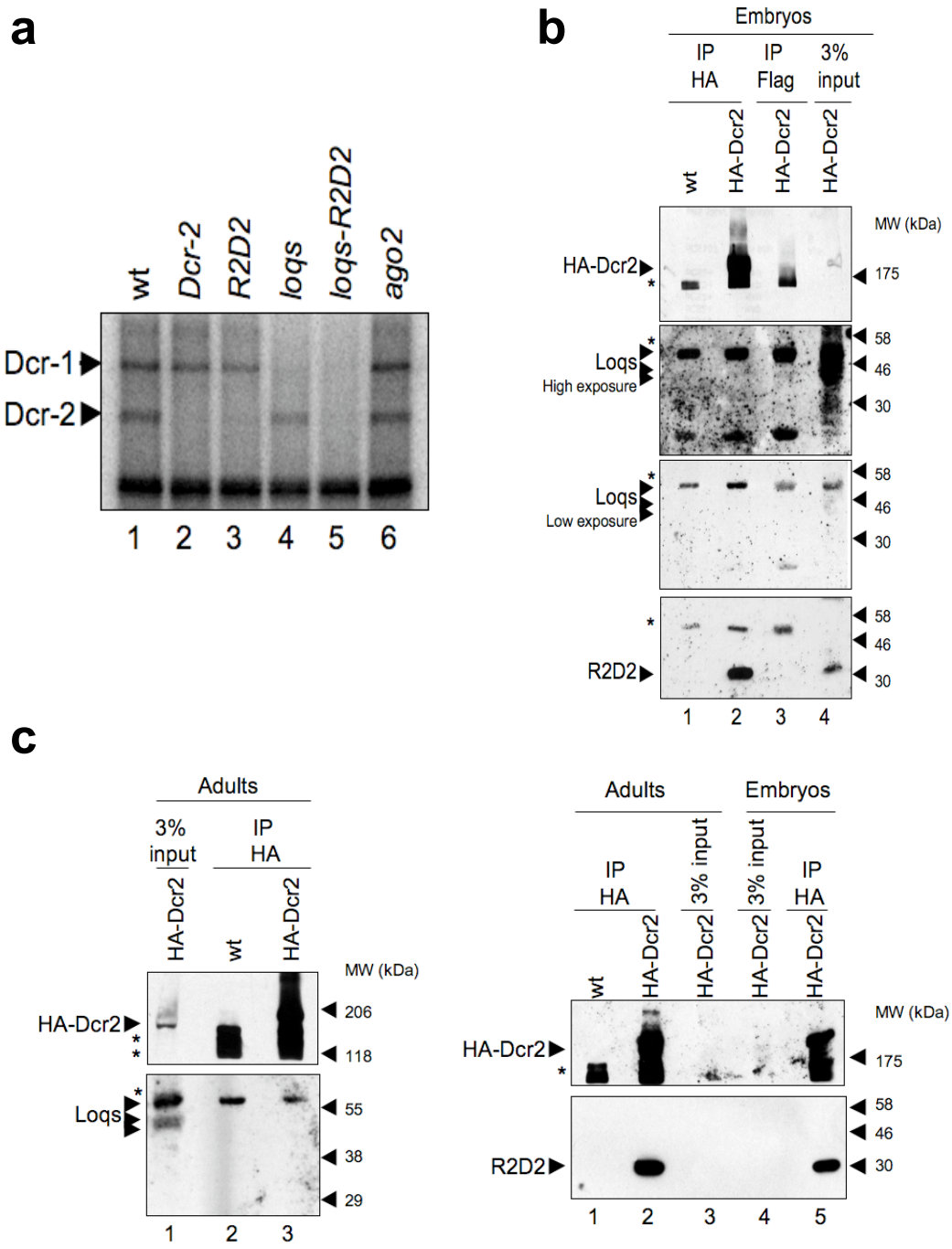
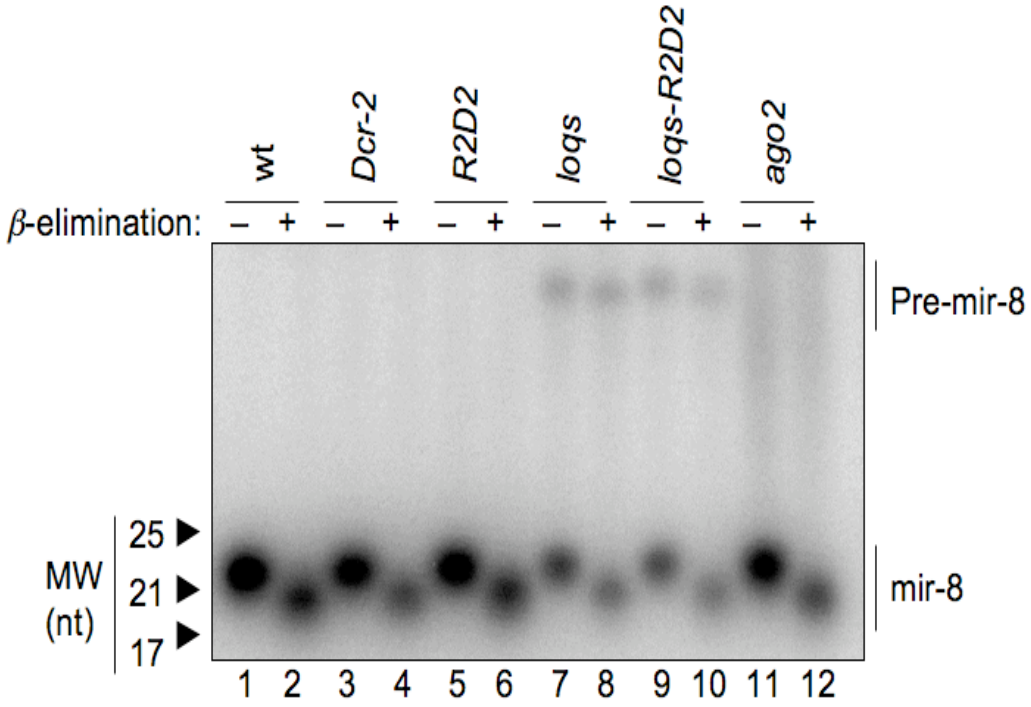
Supplementary Material



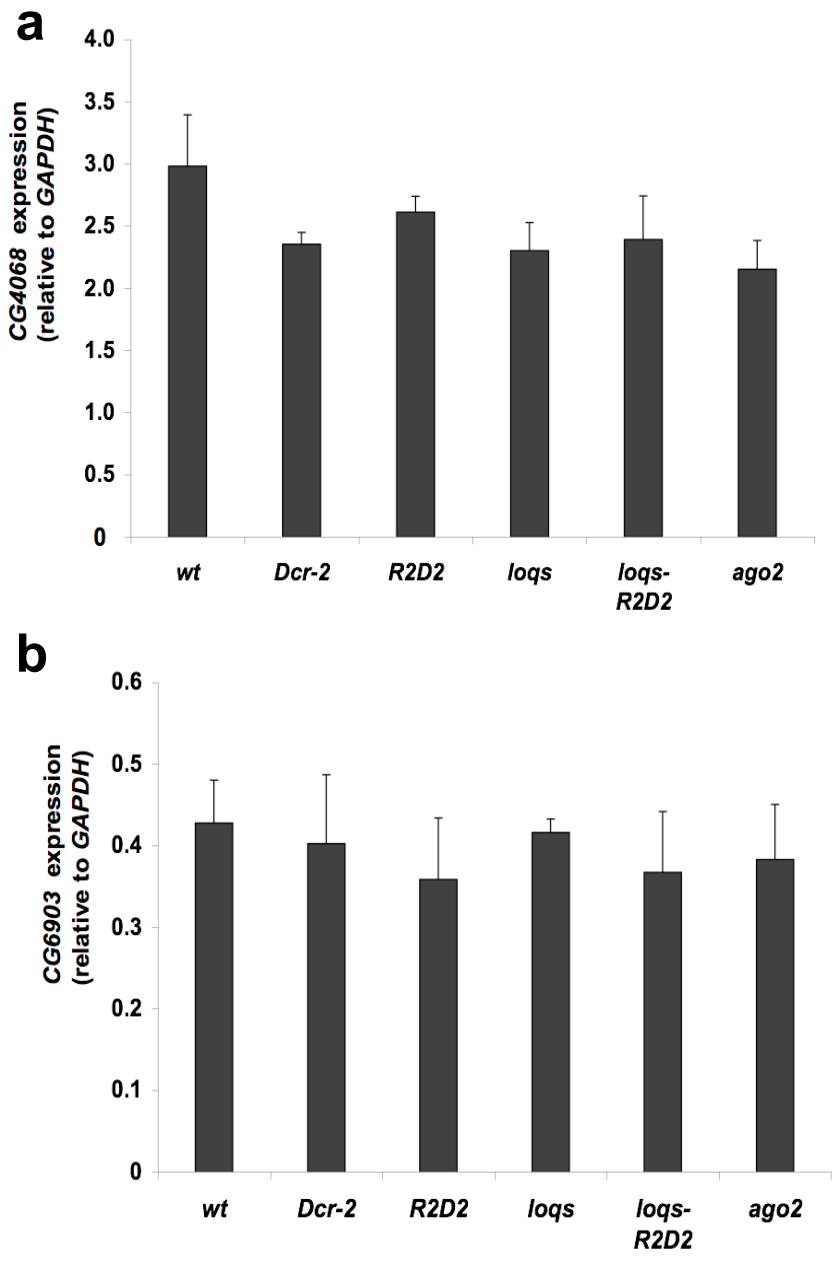
Supplementary Figure 1. The pool of *GMR-wIR* derived siRNAs is changed in *R2D2* mutants compared to wildtype flies. Sequence logo plots of 20 and 22 nt sense (+) and antisense (-) RNAs mapping to *GMR-wIR*.



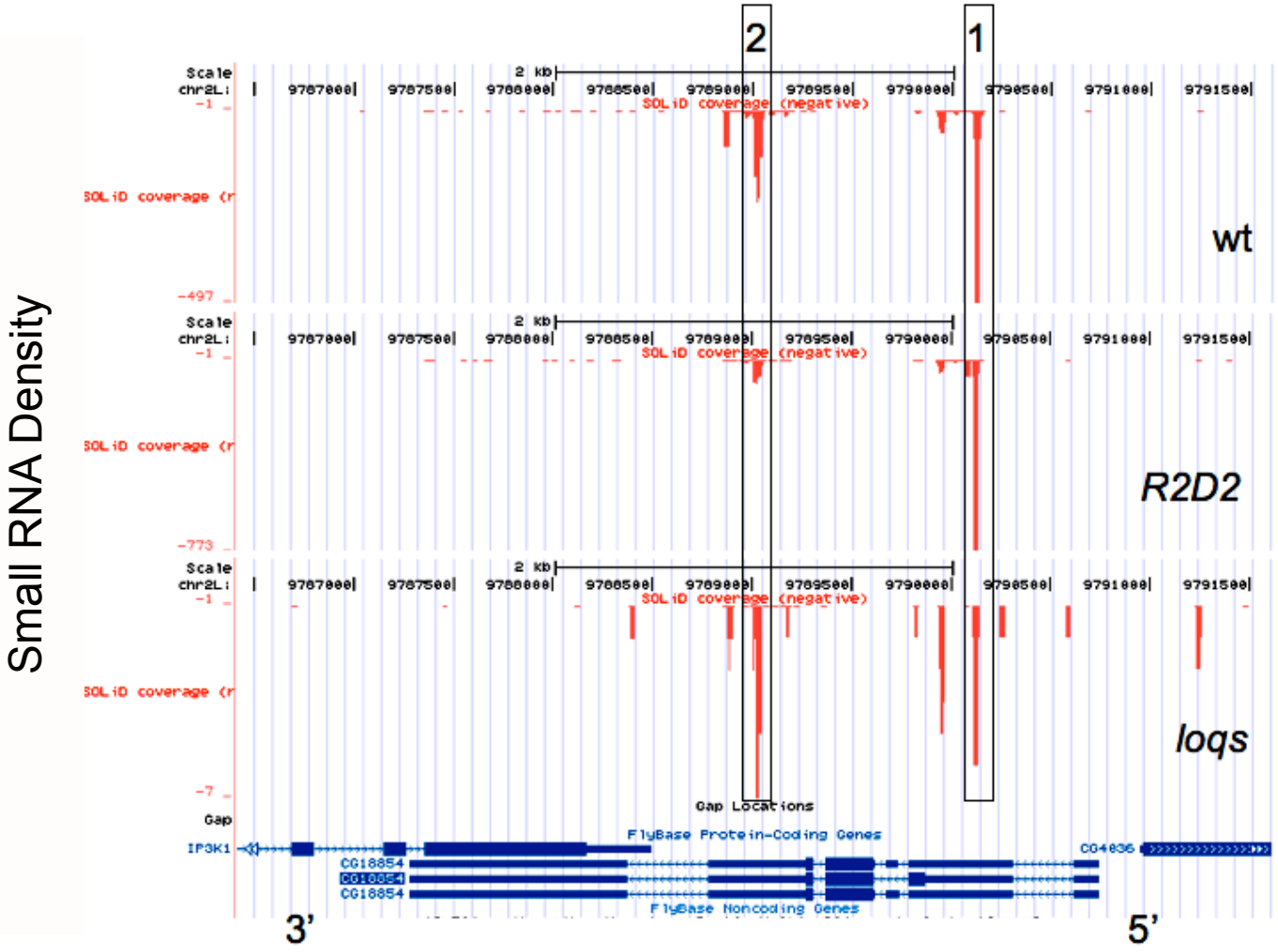
Supplementary Figure 2. R2D2 strongly interacts with Dcr-2. (**a**) siRNA crosslinking to Dcr-2 protein is dependent on R2D2 but not on Loqs. UV crosslinking of a radiolabelled siRNA duplex to Dcr-2 and Dcr-1 proteins in extracts from wildtype and mutant embryos. (**b,c**) Animals expressing HA-tagged Dcr-2 were extracted, and extracts were subjected to anti-HA immunoprecipitation. Pelleted proteins were probed by Western blot for HA-Dcr-2, Loqs and R2D2 proteins. A large percentage of the cellular pool of R2D2 is detected in immunoprecipitates from either embryos (**b**) or adult flies (**c**). None of the Loqs isoforms are detected in the same immunoprecipitates. The asterisk (*) indicates non-specific bands observed in immunoprecipitation samples that includes the heavy chain of the antibodies used that migrates close to the size predicted for Loqs-PB isoform.



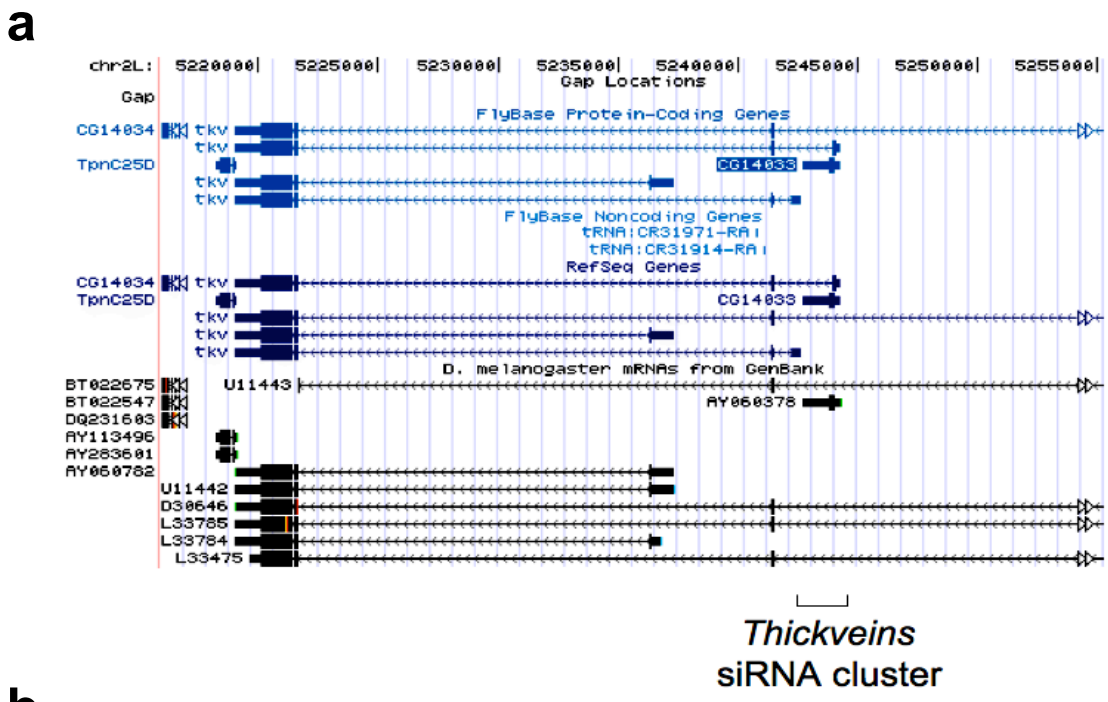
Supplementary Figure 3. Analysis of the 3' end of mir-8 miRNA in different mutants. mir-8 RNA was visualized by Northern blotting from small RNA fractions subjected to β-elimination reactions before electrophoresis.

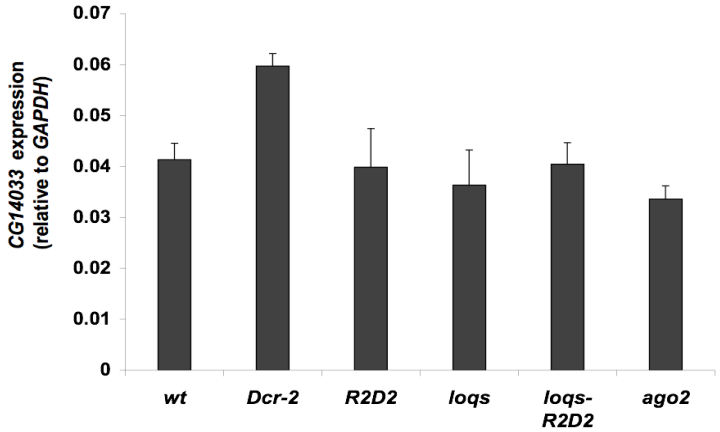


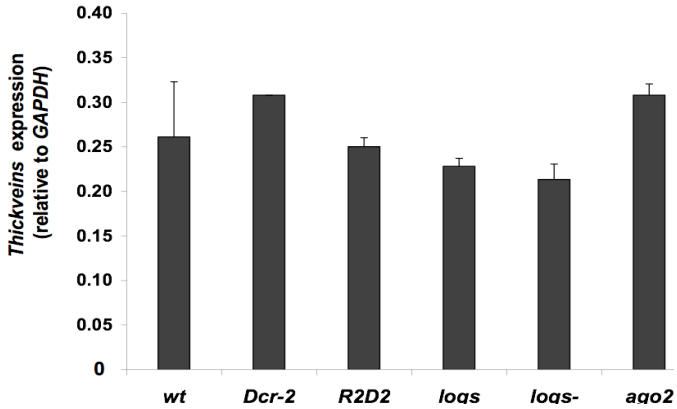
Supplementary Figure 4. Expression of annotated genes surrounding the *esi-2* locus in mutants. (**a**) *CG4068* and (**b**) *CG6903* transcript levels determined by RT-qPCR from total testis RNA. The graphs display the means and standard deviations (*n*=3).



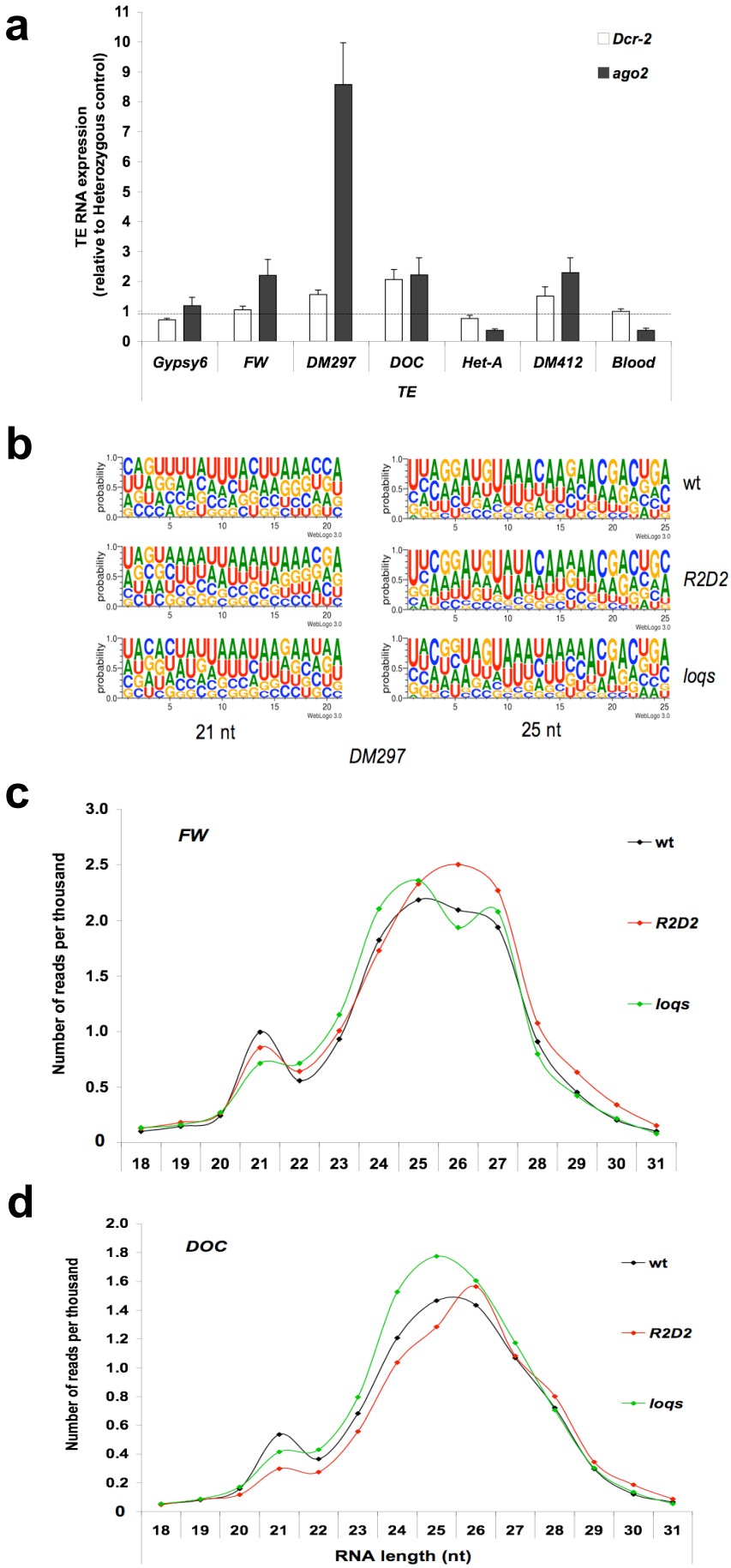
Supplementary Figure 5. Analysis of the esi-RNAs from the *esi-2* locus in wildtype, *R2D2* and *loqs* mutant flies. Plot of the small RNA density profile along the *CG18854* locus, provided by the UCSC Genome Browser. Boxes 1 and 2 highlight the two regions in *CG18854* with highest frequencies of sequenced small RNAs. Note the different scales used for plotting the siRNA density in wildtype, *R2D2* and *loqs* mutants to allow for comparison of the relative coverage.

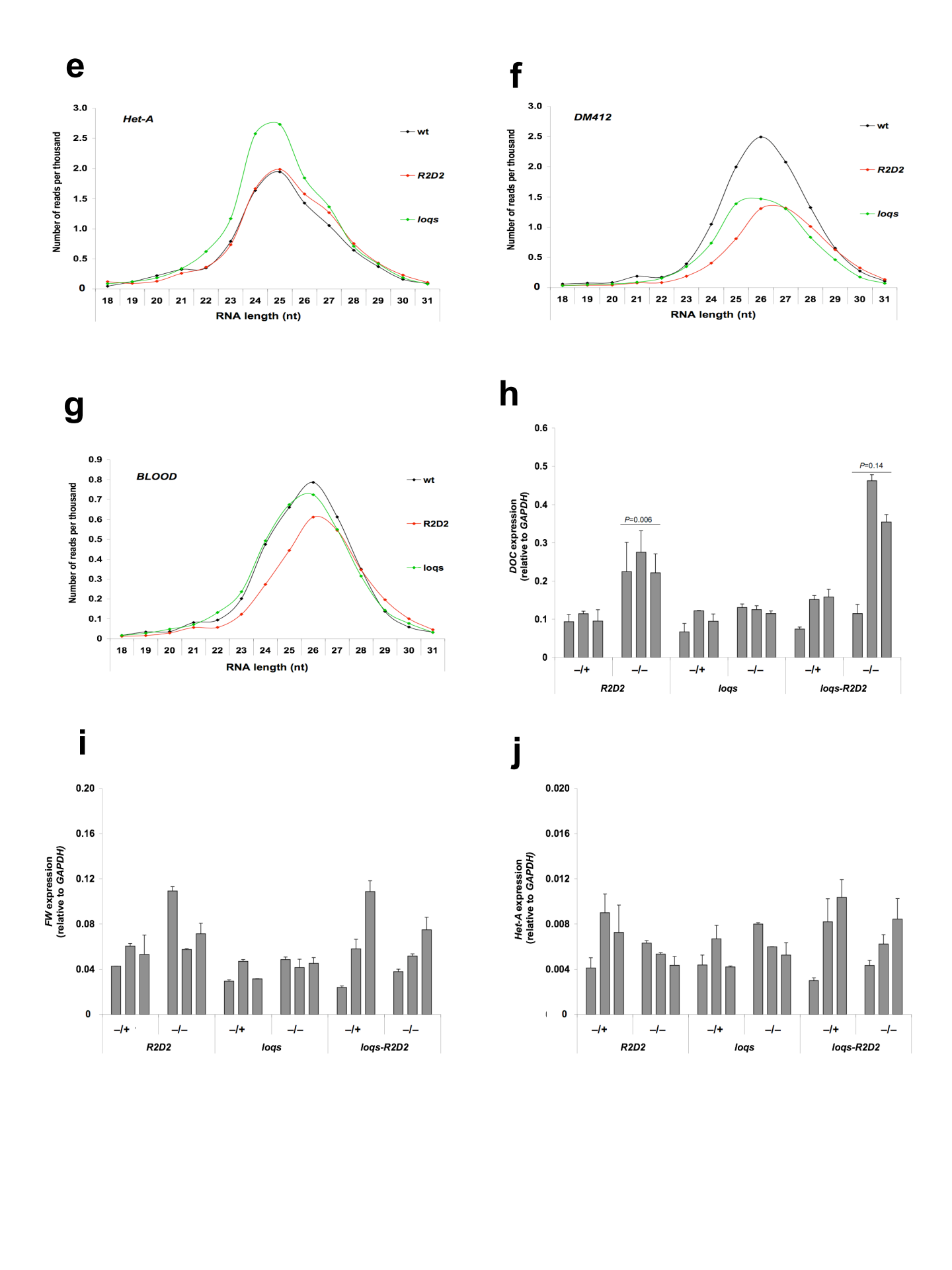


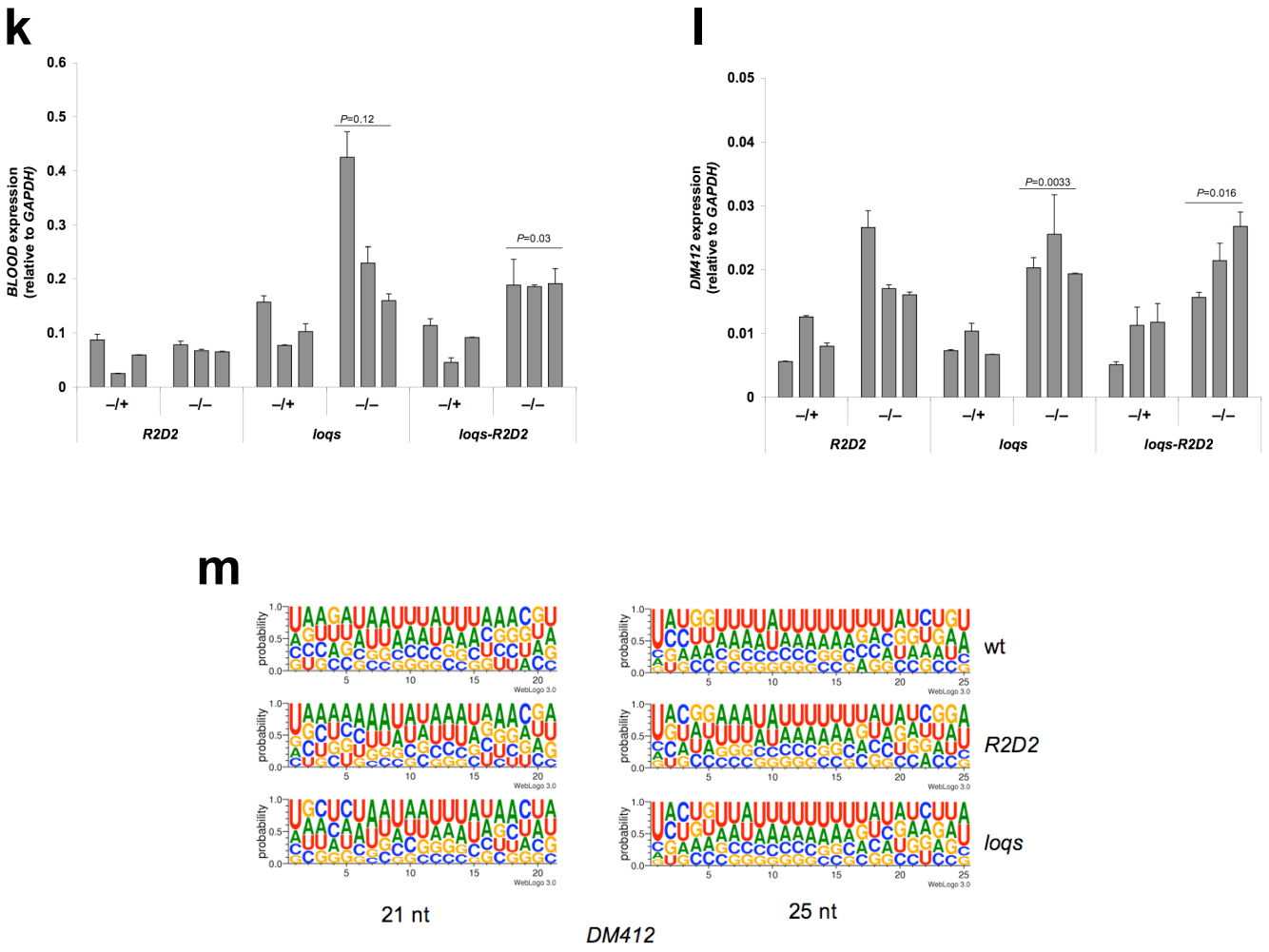




Supplementary Figure 6. Analysis of the *Thickvein* endo-siRNA locus in mutants. (**a**) Graphic representation of the genomic region corresponding to the *Thickveins* endo-siRNA locus on the X chromosome, provided by the UCSC Genome Browser. (**b, c**) *CG14033* (**b**) and *Thickveins* (**c**) transcript levels as determined by RT-qPCR from total testis RNA. The graphs display the means and standard deviations (*n*=3).


a
TE RNA expression
(relative to Heterozygous control)
Dcr-2
ago2
Gypsy6
FW
DM297
DOC
Het-A
DM412
Blood
TE
b
wt
R2D2
loqs
21 nt
25 nt
DM297
c
FW
wt
R2D2
loqs
Number of reads per thousand
d
DOC
wt
R2D2
loqs
Number of reads per thousand
RNA length (nt)


e
Het-A
Number of reads per thousand
RNA length (nt)
wt
R2D2
loqs
f
DM412
g
BLOOD
h
DOC expression (relative to GAPDH)
P=0.006
P=0.14
–/+
–/–
R2D2
loqs
loqs-R2D2
i
FW expression (relative to GAPDH)
j
Het-A expression (relative to GAPDH)



Supplementary Figure 7. Analysis of RNA expression from TEs in mutants. (**a**) TE RNA levels in *Dcr-2* and *ago-2* mutants normalized to their respective heterozygous controls. TE RNA levels were determined by RT-qPCR from total female RNA. The means and standard deviations represent the ratio between homozygotes/ heterozygotes. (**b**) Sequence logo plots of 21 and 25 nt sequenced RNAs mapping to *DM297*. (**c-g**) Size distribution and normalized number of sequenced small RNAs mapping to specific TEs. *FW* (**c**), *DOC* (**d**), *Het-A* (**e**), *DM412* (**f**) and *BLOOD* (**g**) are shown. (**h-l**) TE RNA levels were determined by RT-qPCR from total female RNA. Means and standard deviations are from three independent experiments. *DOC* (**h**), *FW* (**i**), *Het-A* (**j**), *BLOOD* (**k**) and *DM412* (**l**) are shown. Statistically significant differences are indicated (Student *t* test compared to heterozygous controls). (**m**) Sequence logo plots of 21 and 25 nt sequenced RNAs mapping to *DM412*.

Supplementary Table 1. Summary of deep sequencing RNAs

		Wildtype		*R2D2*		*loqs*	
total reads		25,072,677		24,196,794		23,566,713	
adaptors		13,292		9,328		33,219	
mappable reads	**total**	8,921,715		9,512,340		6,279,042	
	rRNA	5,746,501		7,386,817		4,518,217	
mappable minus rRNA	**total**	3,175,214	**%**	2,125,523	**%**	1,760,825	**%**
	miRNA	477,978	15.1	398,234	18.7	163,906	9.3
	mRNA	252,784	8.0	117,143	5.5	151,587	8.6
	TEs	983,863	31.0	645,411	30.4	567,489	32.2

Supplementary Table 2. List of qPCR primers

Gene	Primers	
DM297	Left 991;aaggaacttggacggacaaa	Right 1052;taggccaatcttcgacgttc
Gypsy6	Left 2196 ;ggtacacagtcgcaacatcg	Right 2260;cattgggtattgcttcctatcc
FW	Left 722 ;cagatttgccttgctggtaga	Right 798;gctttgttttcttcggttcg
BLOOD	Left 637, gaccaaagcccttgaccata	Right 717, ggccacccctcttctttta
Het-A	Left 2930 ;gagcaaatcaattgccgaag	Right 2990;gaatggatttaacatcgactttctg
DOC	fwd;taccttaaacaaacaaacatgcccacc	rev;tttgtatgggtggtcagcttttcgT
DM412	Left 2918;agtaatccaagaacagggcaac	Right 3039;cctgttttggcatgaatgaa
Mus308	fwd: aaggattagcgccaagctggaggat	rev: accacgaccactgccacagagattc
CG9203	fwd: agctggcagaaaaaccatgaccagt	rev: caattcttttggcgtagcttgagca
GAPDH	fwd;tgatgaaattaaggccaaggttcagga	rev;tcgttgtcgtaccaagagatcagcttc
Bcd	left 611,cggatctgtcagcgaaacta	right 675,ccgacggttcttaaaccatatc
AY119020	left 1082;caaacacccacacacatacaca	right 1166;ccagggcgctacattcaata
CG18854	Left 940;ggtgctgcgcataccttt	Right 1062;caaggctagggctcgtca
RpL32	Forward; gacgcttcaagggacagtatctg	Reverse; aaacgcggttctgcatgag
CG6903	left 547;tgtacctgcgctcggagt	right 631;ttcactttggtatacggacacg
CG14033	left 409;accaggggaactgatttcg	right 468;tgtgcctgggttttgcttat
Thickvein	left 337;gatgagacgaccgggatgta	right 429;agcggctaccttgcacat
CG4068	left 1538;agagcgcaagtcggtcag	right 1628;caggccgctgactaggatta

Supplementary Methods.

Immunoprecipitation. 1 mL of frozen embryos or adult flies were homogeneized in 2 mL of lysis buffer (35 mM Tris pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$, 0.3% Triton X-100, 30% glycerol) using a dounce homogeneizer. The lysate was centrifuged at 12,000 g for 10 minutes, and the supernatant was filtered through a 0.22 μM syringe filter. Protein concentration was determined using the Bradford assay (Biorad). 5-10 mg of total lysate protein was incubated with anti-HA or anti-Flag beads (Sigma) at 4° C. Beads were pre-equilibrated in lysis buffer before being added to the lysate. After 16 hours, beads were centrifuged and washed 6 times in wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$, 0.1% NP-40). Beads were boiled for 5 minutes in protein loading buffer and the supernatant was fractionated using SDS-PAGE. Proteins were transferred to nylon membranes and proteins were detected by Western blotting.

UV Crosslinking. ^{32}P 5' labeled siRNA duplexes were incubated for 30 min at 25° in a 10μl standard RNAi reaction mixture. The reactions were placed onto a parafilm-covered, pre-chilled, metal block in ice and exposed to 254 nm light (at full power) for 30 min in a Stratalinker 2400 (Stratagene). The reactions were then analyzed by 5% SDS-PAGE.